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Abstract

Differential, histochemical and immunohistochemical changes were observed in hepatocytes from immediately to 7 days after isoflurane or sevoflurane exposure (at H 0 to on Day 7) to study the process of development and recovery in anesthetic-induced hepatic injury. A total of 570 7-week-old male Sprague-Dawley rats with or without phenobarbital treatment were exposed to isoflurane or sevoflurane in 100%, 21%, or 10% oxygen, or to 10% oxygen alone for 2h. In phenobarbital-treated rats, hepatocytes both with and without anesthetic exposure markedly changed in 10% oxygen at H 0. Glycogen and ribosomal ribonucleic acid (rRNA) disappeared at H 0 and at H 6, respectively, and at H 6, AST levels in the blood rose. From H 6 to Day 1, necrosis developed more markedly and widely in zone 3 hepatocytes exposed to anesthetics in 10% oxygen than in those exposed to oxygen alone. All degenerated tissues had returned to normal levels by day 7. Recovery of the hepatolobular structure may be attributed to rearrangement of remaining hepatocytes in the portal vein area. Both the disappearance of glycogen and rRNA and the increase in blood AST levels after exposure to isoflurane or sevoflurane are considered to be factors contributing to the induction of necrosis around the central vein. The grade of isoflurane-induced hepatic injury was found to be significantly higher than that of sevoflurane.

KEYWORDS: isoflurane, sevoflurane, histochemistry, hypoxia, hepatic injury

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Original Article

Differential, Histochemical and Immunohistochemical Changes in Rat Hepatocytes after Isoflurane or Sevoflurane Exposure

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Differential, histochemical and immunohistochemical changes were observed in hepatocytes from immediately to 7 days after isoflurane or sevoflurane exposure (at H 0 to on Day 7) to study the process of development and recovery in anesthetic-induced hepatic injury. A total of 570 7-week-old male Sprague-Dawley rats with or without phenobarbital treatment were exposed to isoflurane or sevoflurane in 100%, 21%, or 10% oxygen, or to 10% oxygen alone for 2 h. In phenobarbital-treated rats, hepatocytes both with and without anesthetic exposure markedly changed in 10% oxygen at H 0. Glycogen and ribosomal ribonucleic acid (rRNA) disappeared at H 0 and at H 6, respectively, and at H 6, AST levels in the blood rose. From H 6 to Day 1, necrosis developed more markedly and widely in zone 3 hepatocytes exposed to anesthetics in 10% oxygen than in those exposed to oxygen alone. All degenerated tissues had returned to normal levels by day 7. Recovery of the hepatobular structure may be attributed to rearrangement of remaining hepatocytes in the portal vein area. Both the disappearance of glycogen and rRNA and the increase in blood AST levels after exposure to isoflurane or sevoflurane are considered to be factors contributing to the induction of necrosis around the central vein. The grade of isoflurane-induced hepatic injury was found to be significantly higher than that of sevoflurane.

Key words: isoflurane, sevoflurane, histochemistry, hypoxia, hepatic injury

Halothane-related hepatic injury has been described in many reports [1, 2], and the products of halothane biotransformation have been suggested to induce hepatic injury [3, 4]. Approximately 20%, 2.0%, and 0.2% of inhaled halothane, enflurane, and isoflurane, respectively, is metabolized, and harmful substances are produced *in vivo* [5]. Sevoflurane is also metabolized *in vivo*, but it does not produce reactive metabolites [6]. Despite these variations, sevoflurane,

isoflurane and enflurane all reportedly cause hepatic injury accompanied by centrilobular necrosis [7–13], though the etiology of the hepatic injury induced by these anesthetics has not yet been clarified.

There have been a few reports of histochemical and immunohistochemical observations of intrahepatocellular changes after exposure to halothane and isoflurane [14–16]. In the current study, the authors histochemically and immunohistochemically observed hepatic changes using the same methods as those used previously [15, 17] to study the differences in the occurrence of early-stage hepatic injury and time-course changes (including development of and recovery from hepatic injury) in the

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rat liver at specific sites from immediately to 7 days after exposures to isoflurane (metabolized *in vivo* to produce harmful substances) and sevoflurane (metabolized, but not producing harmful substances).

Materials and Methods

All animal experiments were performed according to the Guidelines for Animal Experiments of the Okayama University Medical School.

Materials. The materials and reagents used in this study were as follows: 7-week-old male Sprague-Dawley (SD) rats (Clea Japan Inc., Osaka, Japan); sevoflurane and isoflurane (Dainippon Pharmaceutical Co., Osaka, Japan); phenobarbital sodium and pentobarbital sodium (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan); Mayer hemalum solution, and Schiff's reagent (PAS) (Merck, Darmstadt, Germany); 4% paraformaldehyde and 1% eosin Y solution (Muto Pure Chemicals Co., Ltd., Tokyo, Japan); anti-macrophage mouse monoclonal antibody (ED 1) (Serotec Ltd., Oxford, England); diastase (α amylase) and ribonuclease I (Sigma-Aldrich chemie gmbh, Sreinheim, Germany); 5-bromo-2'-deoxyuridine (BrdU), anti-BrdU mouse monoclonal antibody, anti-single-stranded DNA (ssDNA) rabbit polyclonal antibody, anti-mouse or anti-rabbit immunoglobulins conjugated to peroxidase-labeled dextran polymer (EnVision + TM · Peroxidase), and 3,3'-diaminobenzidine (DAB) substrate kit (Dako Japan Co., Ltd., Kyoto, Japan), and IATAROZYME TA-Lq (RM 163-K) (Iatron Laboratories Inc., Tokyo, Japan) as a reagent for analyzing aspartate aminotransferase (AST).

Reagents of the best quality were purchased in addition to those mentioned above.

Animals and Procedures. As shown in Fig. 1, male SD rats were divided into 2 groups: nontreated healthy rats (N-group) and rats given water containing 0.1% phenobarbital solution (PB) *ad libitum* for 5 days (PB-group) before the experiment [15, 17]. The experiment was carried out as follows: Rats from both groups were separately exposed to 1.5% isoflurane and 2.0% sevoflurane (approximately 1 minimum alveolar concentration [MAC] of isoflurane [18] and sevoflurane [19]), respectively, in 100%, 21%, or 10% oxygen, or 10% oxygen alone without anesthetics for 2 h. Air was used for 21% oxygen, and air and nitrogen were mixed at a 1:1 ratio with an anesthesia apparatus (Model CK-70, Acoma Co., Tokyo, Japan) to create gas

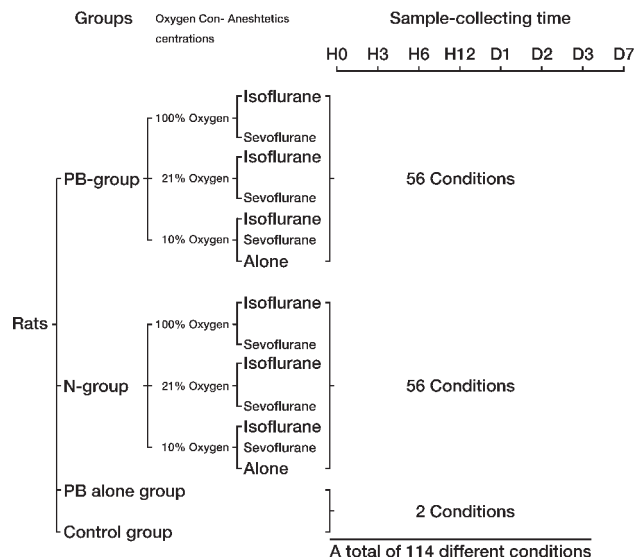


Fig. 1 Grouping of rats and sample-collecting time. The total number of conditions and rats examined were 114 conditions and 570 rats (5 rats each were used under each condition). PB-group, rats pretreated with 0.1% phenobarbital solution (PB) *ad libitum* for 5 days. N-group, rats without PB pretreated. PB alone-group, rats treated with PB alone. Control group, nontreated healthy rats. H 0–12 and D 1–7 show the sample-collecting time at 0 (immediately)–12 h and 1–7 days after each anesthetic exposure.

containing 10% oxygen. The flow-volume of oxygen gas of 3 different concentrations (100%, 21%, and 10%) was set at 4 L/min with the anesthesia apparatus. Concentrations were adjusted to 1.5% and 2.0% for isoflurane (Penlon Ltd., Abingdon, UK) and for sevoflurane (Akoma Co., Tokyo, Japan), respectively, with vaporizers. Rats in a polyethylene container (20 × 30 × 10 cm) were exposed to isoflurane or sevoflurane through a tube connected to the vaporizer.

The livers of 5 rats from each exposure group were collected at 0 h (immediately), 3 h, 6 h, 12 h, 1 day, 2 days, 3 days, and 7 days after the isoflurane or sevoflurane exposure (at H 0–12, on Days 1–7). Five rats each were used under 114 different conditions, *i.e.*, 8 different collection times by 3 different 2-h exposures (in 100%, 21%, or 10% oxygen) to 2 different anesthetics, or in 10% oxygen alone in 2 groups, plus the group of rats treated with PB alone, and 1 control (untreated) group (Fig. 1).

The collected livers were histochemically and immunohistochemically studied for general cell structure, glycogen, apoptosis, ribosomal ribonucleic acid (rRNA) gran-

ules in the cells, cell proliferation, and infiltrated cells. When the livers were collected, 5 specimens, each taken under different test conditions, underwent a median incision in the abdomen under the anesthesia with pentobarbital sodium. Next, 3 ml of the blood was collected in a heparinized glass syringe from the left ventricle to determine the AST measurement.

Morphological and Immunohistochemical Studies. The liver was excised after perfusion through the portal vein with 4% paraformaldehyde (4% PFA), cut into pieces in 2–3 mm thick, and fixed in 4% ice-cooled PFA. After being dehydrated with alcohol according to routine procedures, the sections were embedded in paraffin, cut into pieces 5 μ m thick, stained with H-E, PAS, and methylgreen pyronin (MGP), and examined by diastase (α amylase) and ribonuclease digest tests.

Arbitrarily selected paraffin-removed sections were immersed in 3% H_2O_2 /MtOH at room temperature for 10 min to dispose of the intrinsic peroxidase, then immersed in trypsin solution (1 mg trypsin/ml 0.1 M PBS) at 37 °C for 30 min to identify the infiltrated and apoptotic cells. Next, ED 1 and anti-ssDNA antibodies (rabbit) were used as the primary antibodies to identify infiltrated and apoptotic cells. EnVision + TM • peroxidase (mouse or rabbit) were used as the secondary antibodies to make the target cells emit colors with a DAB substrate kit. The spleen and small intestine were used to test for positive reactions to macrophage (ED 1) and apoptosis (ssDNA).

To study hepatocellular proliferation, BrdU (50 mg/kg) dissolved in 1 ml of PBS was intraperitoneally administered 2 h before liver resection. The rats underwent median abdominal incision under pentobarbital anesthesia. The liver was then excised, immersed in 70% ice-cooled ethanol, dehydrated, and embedded in paraffin. After the liver was cut into 5- μ m sections, the slide glasses were immersed in 3% H_2O_2 /MtOH. Anti-BrdU mouse monoclonal antibody was used for the primary antibody, and EnVision + TM • peroxidase (mouse) were used for the secondary antibodies. The remaining one-third of the liver was used for the BrdU-intake test (proliferation) by the methods of Higgins and Anderson [20].

Numerical Analysis of Serum AST Values and the Area Negatively Stained with PAS after Anesthetic Exposure. AST values from H 0 to Day 7 after exposure to isoflurane or sevoflurane were analyzed in the serum collected from 5 rats by the

statistical tests described below to compare them with the values of the control group and with values collected at different times. The data were analyzed by a Dunnett's test to compare with control values to get a significant difference, and by a Tukey's honestly significant difference test to compare among collection times. Values of $P < 0.05$ were assumed to be significant.

The area of the lobule negatively stained with PAS [PAS(–)] is expressed as a percentage of the liver lobule. The length of the lobule from the central vein (CV) to the portal vein (PV) was measured as a , and that of the PAS(–) area from the CV to PV as b to calculate the PAS(–) area as a percentage of the lobule (y) as follows: $y(\%) = b/a \times 100$. Straight lines of the sinusoid running from the PV to the CV were selected to measure the lengths of a and b . At each collection time, a and b were measured in the 25 samples that had been taken from 5 different regions in the lobules of 5 rats. The mean percentage of the PAS(–) area (Y) was obtained from these 25 (y) samples (Y : mean \pm SD). Each value of Y was analyzed by the same statistical tests described above to compare it with the H 0 values and to compare among values at different collection times.

Results

Time-Course Changes in Serum AST Values after Anesthetic Exposures. As shown in Fig. 2, the AST values (mean \pm SD) of rats in the control and PB-pretreated alone groups were 46.0 ± 5.2 U and 47.4 ± 6.9 U, respectively. The AST values in both the PB- and the N-groups after isoflurane or sevoflurane exposure under 100% and 21% oxygen, or exposure to 10% oxygen alone, differed little from those of the control group (data not shown).

In the PB-group, the AST values differed from those of the control group after each anesthetic exposure under 10% oxygen. From H 3 after each anesthetic exposure under 10% oxygen, the AST values began to increase and at H 6 significantly increased (isoflurane: 91.6 ± 13.5 U and sevoflurane; 84.4 ± 8.3 U) when compared with those of the control group ($P < 0.01$), reaching the highest value at H 12 (isoflurane; 98.6 ± 10.0 U and sevoflurane; 91.2 ± 11.0 U) ($P < 0.01$). On Day 1, the AST values began to decrease, returning to normal levels by Day 3. In the N-group, the AST values were slightly higher than those of the control group from H 3 to Day 1, reached a peak (isoflurane: 68.0 ± 12.6 U,

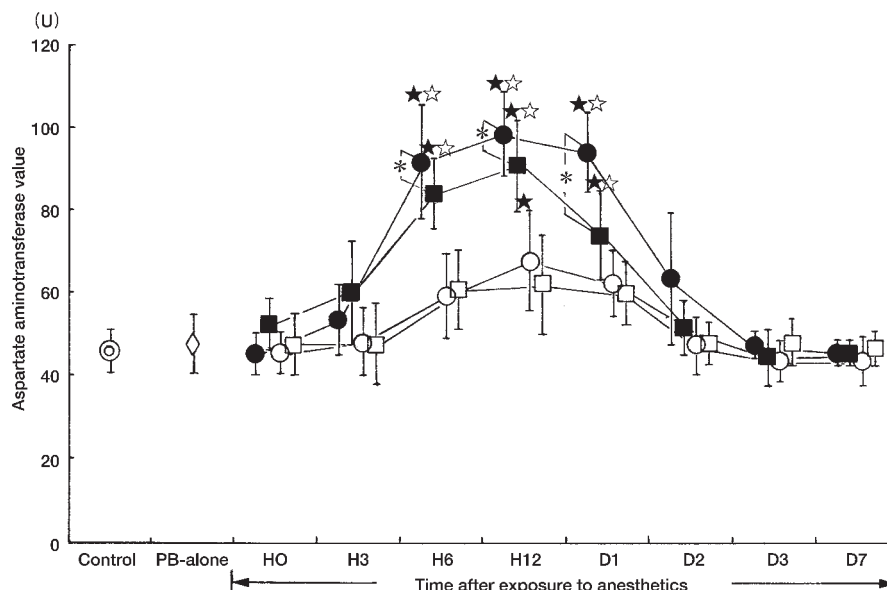


Fig. 2 Time-course changes in serum aspartate aminotransferase (AST) after anesthetic exposure under 10% oxygen. Each collecting value was the mean \pm SD of 5 rats' values. \odot , Control group (Control); \diamond , PB alone group (PB-alone); \bullet , Isoflurane exposure in the PB-group; \blacksquare , Sevoflurane exposure in the PB-group; \circ , Isoflurane exposure in the N-group; \square , Sevoflurane exposure in the N-group; \star , significantly higher than that of the control ($P < 0.01$); $*$, significant differences among the values after isoflurane and sevoflurane exposures ($P < 0.05$); \star , significant differences among the values in the PB- and N-groups after exposure to each anesthetic ($P < 0.01$); at H 0 - H 12 and on Day 1 - Day 7, see Fig. 1.

sevoflurane: 62.2 ± 12.2 U) at H 12, and then returned to normal levels on Day 2 after each anesthetic exposure.

From H 6 to Day 1, AST levels were significantly higher in isoflurane-exposed cells than in those exposed to sevoflurane ($P < 0.05$), and there were significant differences among the values between the BP- and the N-groups after exposure to each anesthetic ($P < 0.01$).

Histochemical Changes. Figs. 3, 4, and 5 show the changes caused by PAS, MGP, and H-E stainings from the control to Day 1. Fig. 6 shows the statistical analyses of changes in the PAS(−) area under 10% oxygen concentration from H 0 to Day 7.

An image of the liver tissues in control rats is shown in Figs. 3a, 4a, and 5a. PAS-positive substances (Fig. 3a) and MGP-positive granules in the cells (Fig. 4a) were observed evenly in the area from the CV to the PV. The photos in the right lower portions of Figs. 3a and 4a show the results of the diastase or ribonuclease digest test, and that the positively stained granule with PAS and MGP is that of glycogen or rRNA. Figure 5a shows an image of the liver tissues stained with H-E that is almost identical to that of rats pretreated with PB alone, as seen in the control rats (Figs. 3e, 4e, and 5e).

In the N-group, no histological changes were seen in any of the hepatocytes exposed to the anesthetics for 2 h under 100% or 21% oxygen compared with the control images. However, after 2 h exposure to isoflurane or sevoflurane in 10% oxygen, PAS staining slightly decreased to approximately 35% of the area from the CV to the PV in response to both of these anesthetic exposures, respectively, decreasing significantly from H 0 to H 6, then returning to the control level at H 12 (Fig. 6).

In the PB-group, there were no changes in PAS staining under 100% oxygen as compared with the control group. However, PAS staining decreased to approximately 30–35% of the area from the CV to the PV after 2 h of exposure to isoflurane or sevoflurane in 21% oxygen at H 0, but then increased again in a manner similar to that of the N-group in 10% oxygen alone. Changes in cell structure could not be identified by H-E or MGP staining in the area where the PAS staining decreased.

Marked changes were observed in the cells exposed to isoflurane or sevoflurane for 2 h in 10% oxygen. By PAS staining, after isoflurane exposure, approximately 36.5% of the area from the CV to the PV was revealed

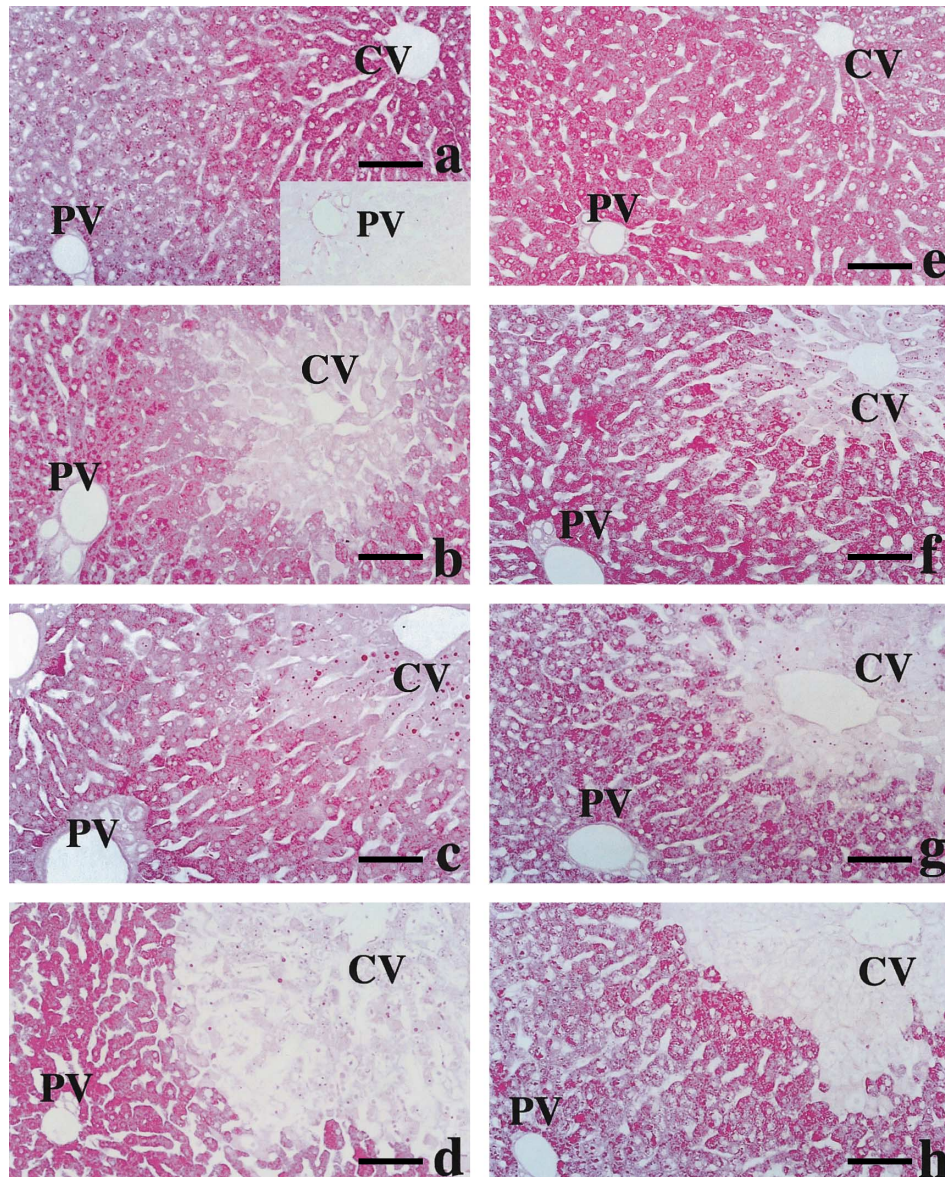


Fig. 3 Changes in the hepatocytes stained with periodic acid Schiff(PAS) after isoflurane or sevoflurane exposure under 10% oxygen in the PB-group. **a**, PAS-stained image of the control hepatocytes. PAS-positive reaction is evenly seen from the central vein (CV) to the portal vein (PV). The photo, in the right lower portion shows the results of the diastase digest test. **b**, **c**, **d**, Hepatocytes exposed to isoflurane at H 0(**b**), H 6(**c**), and H 12(**d**). At H 0, H 6, and H 12, the negative PAS-staining [PAS(-)] area spread to approximately 36.5%, 47.6%, and 51.4% of the area, respectively, from the CV to the PV. **e**, PAS-stained image of the hepatocytes treated with PB alone. **f**, **g**, **h**, Hepatocytes exposed to sevoflurane at H 0(**f**), H 6(**g**), and H 12(**h**). At H 0, H 6, and H 12, the PAS(-) area is seen in approximately 33.8%, 35.3%, and 36.8% of the area, respectively, from the CV to the PV. At H 6 after exposure to isoflurane or sevoflurane, cell-swelling, pyknosis, and fusion began to appear in the PAS(-) area. At H 12, these cell-degenerative changes in the cells reached a peak. Bars in photos a-h indicate 20 μ m

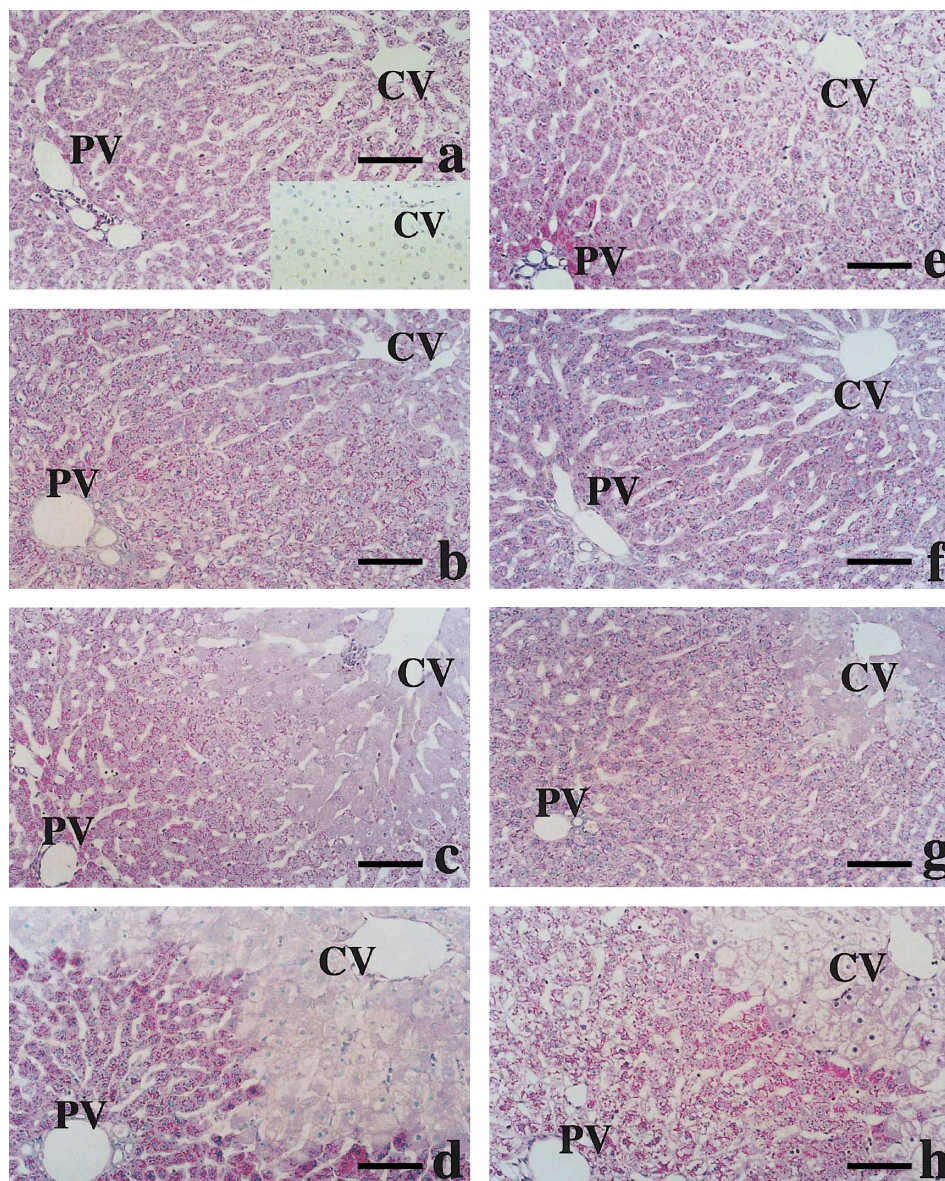


Fig. 4 Changes in the hepatocytes stained with methylgreen pyronin (MGP) after isoflurane or sevoflurane exposure under 10% oxygen in the PB-group. **a**, MGP-stained images of the control hepatocytes. rRNA granules positively stained with MGP are evenly seen from the CV to the PV. The photo, in the right lower portion shows the result of the ribonuclease digest test and that rRNA granule was positively stained to MGP. **b**, **c**, **d**, Hepatocytes exposed to isoflurane at H 0(**b**), H 6(**c**), and H 12(**d**). **e**, MGP-stained image of the hepatocytes treated with PB alone. **f**, **g**, **h**, Hepatocytes exposed to sevoflurane at H 0(**f**), H 6(**g**), and H 12(**h**). At H 0 after exposures to both isoflurane and sevoflurane, rRNA granules positively stained with MGP are evenly seen in the cells from the CV to the PV, as seen in the control. At H 6, rRNA granules have disappeared from the cells in the PAS(−) area. At H 12, the rRNA granule has completely disappeared from the cells in the PAS(−) area. Bars in photos a-h indicate 20μm. PAS(−), see Fig. 3.

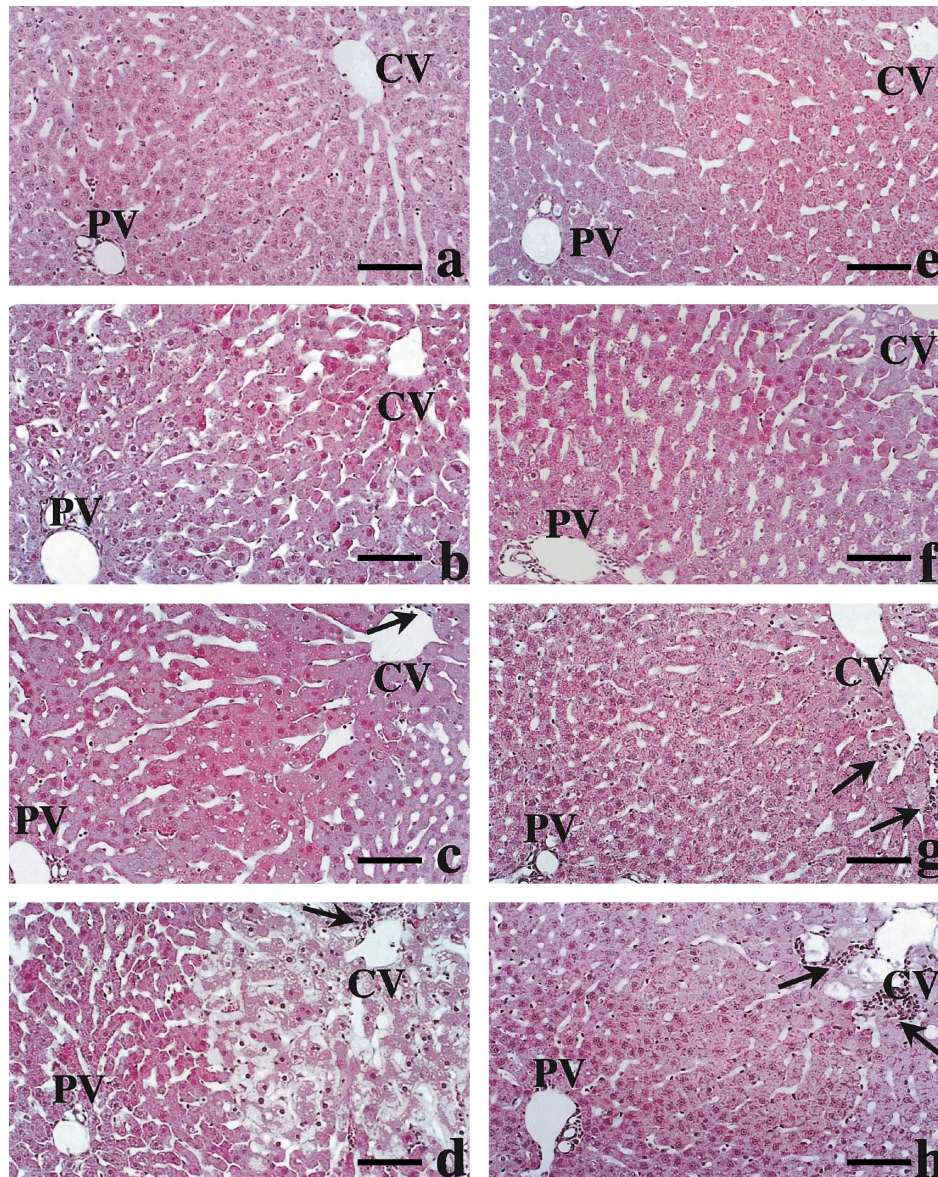


Fig. 5 Changes in the hepatocytes stained with H-E after isoflurane or sevoflurane exposure under 10% oxygen in the PB-group. **a, e**, H-E-stained images of the control hepatocytes (**a**) and of those treated with PB alone (**e**). The image of the hepatocytes treated with PB alone is almost identical to that of the control group. **b, c, d**, Hepatocytes exposed to isoflurane at H 0 (**b**), H 6 (**c**), and H 12 (**d**). **f, g, h**, Hepatocytes exposed to sevoflurane at H 0 (**f**), H 6 (**g**), and H 12 (**h**). At H 0 after exposures to both isoflurane and sevoflurane, the images are almost identical to that of the control group. At H 6, cells in the PAS(–) area are swollen, and vacuoles, pyknosis, and cell fusion have appeared. The cells are homogeneously stained with eosin. The infiltrated cells (↑) have begun to disperse in the PAS(–) area. At H 12, cells in the PAS(–) area are swollen, and pyknosis, cell fusion and denucleation are visible. Large vacuoles have appeared in the PAS(–) areas. Many infiltrated cells are visible in the PAS(–) area (↑). Bars in photos a-h indicate 20μm. PB-group, see Fig. 1; PAS(–), see Fig. 3.

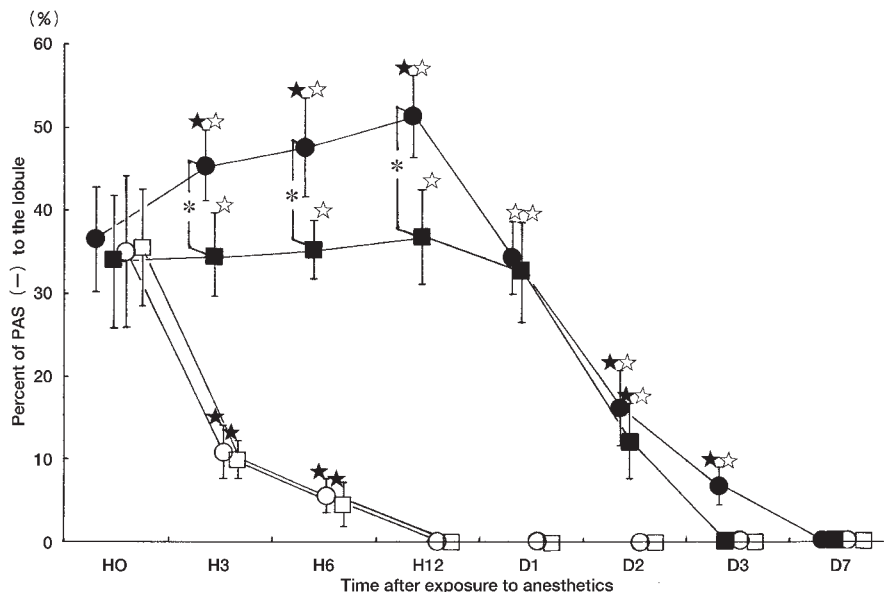


Fig. 6 Time-course changes in the area from the CV to the PV where the liver cells have become PAS(−) after isoflurane or sevoflurane exposure in the PB- and N-groups under 10% oxygen. Each value was obtained from the mean \pm SD of 25 values of each sample. Each sample value (y) is the percentage of the PAS(−) (b) to the length from the CV to the PV (a): $y = b/a \times 100$. ●, isoflurane exposure in the BP-group; ■, sevoflurane exposure in the BP-group; ○, isoflurane exposure in the N-group; □, sevoflurane exposure in the N-group; ★, significantly larger or smaller than that at H 0 ($P < 0.01$); ☆, significant differences among the values in the PB- and N-groups ($P < 0.01$); *, significant differences among the values after isoflurane and sevoflurane exposures ($P < 0.01$); at H 0 - H12 and on Day 1–Day 7. In the N-group, PAS staining area significantly decreased until H 6 after sevoflurane (□) or isoflurane (○) exposure under 10% oxygen, and recovered to the normal level at H 12. H 0–H12 and D 1–7, see Fig. 1; PB- and N-group, see Fig. 1; PAS(−), see Fig. 4.

to be PAS(−) at H 0 (Figs. 3b and 6). The PAS(−) area increased beginning at H 3, reached a peak at H 12, covering approximately 51.4% of the area from the CV to the PV (Figs. 3d and 6), which was a significant increase compared with that at H 0, and decreased from Day 1, returning to normal by Day 7 (Fig. 6). After a 2 h sevoflurane exposure in 10% oxygen, the PAS(−) area was observed to cover approximately 33.8% of the area from the CV to the PV at H 0 (Figs. 3f and 6). Slightly increasing beginning at H 3, the PAS(−) area reached a peak at H 12, covering approximately 36.8% of the area from the CV to the PV (Figs. 3h and 6), but it did not change significantly compared with that of H 0 and decreased from Day 1, then returning to normal by Day 3 (Fig. 6). In the PAS(−) area at H 6 after exposure to isoflurane or sevoflurane, signs of cell-swelling, pyknosis, and fusion began to appear with these degenerative changes reaching a peak at H 12. From H 6 to H 12 after the sevoflurane exposure, the size of the PAS(−) area was significantly smaller than that after isoflurane exposure ($P < 0.01$) (Figs. 3c, d, g, h, and Fig. 6).

By MGP-staining it was found that distribution of rRNA granules began to disappear from 2–3 layers in the PAS(−) cells around the CV at H 3. During the period from H 6 to Day 3 (isoflurane), and from H6 to Day 2 (sevoflurane), rRNA granules completely disappeared in the PAS(−) cells (Figs. 4c, d and Figs. 4g, h), then returned to normal levels by Day 7 (isoflurane) and Day 3 (sevoflurane) (Fig. 6).

By H-E staining, the image was not morphologically different from that of the control group in the PAS(+) area; cells in the PAS(−) area, however, were homogeneously stained with eosin from H 6 to Day 3 (isoflurane) and from H 6 to Day 2 (sevoflurane) (Figs. 5c, d, g, and h), then returned to normal by Day 7 (isoflurane) and Day 3 (sevoflurane). From H 6 to Day 1, cells in the PAS(−) area were swollen with signs of fusion, vacuoles, pyknosis, and denudation (Figs. 5d and h). At H 6, the infiltrated cells began to disperse in the PAS(−) area, and at H 12 many infiltration cells were visible (Figs. 5c, d, g, and h).

In the N-group, no changes were seen in the liver

tissues after a 2-h exposure to 10% oxygen alone. In the PB-group, however, PAS(−) cells appeared in zone 3 at H 0 after such exposure. In this PAS(−) area, the cells were slightly swollen, rRNA granules appeared in the cells at H 6, and vacuolated cells appeared at H 12, while rRNA granules had completely disappeared at H 12, but the tissue recovered to its normal hepatolobular structure on Day 2. Significant differences were found in the size of the PAS(−) area at each collection time between the PB- and the N-groups from H 3 to Day 2 after each anesthetic exposure (Fig. 6).

To summarize Figs. 2 and 6, the PAS(−) area covered approximately 36.5% and 33.8% of the isoflurane- and sevoflurane-exposed livers, respectively, from the CV to the PV at H 0. At H 6, the PAS(−) area increased to approximately 47.6% (isoflurane) and 35.3% (sevoflurane) of the livers from the CV to the PV, respectively, and rRNA in the cells disappeared from the PAS(−) area. From this time forward, AST levels, which had been 46 U in the control, began to significantly increase to approximately 91.6 U (isoflurane) and 84.4 U (sevoflurane), respectively. After the PAS(−) area and AST levels in the blood continued to increase until H 12, both began to decrease and recovered to the control levels on Day 3.

Immunohistochemical Observation.

Infiltrated cells began to appear in the PAS(−) area in the PB-group from H 6 after both anesthetic exposures and were cross-reacted with ED 1, which indicated that they were macrophages. The reaction of the BrdU- or ssDNA-intake was not observed until Day 7, indicating that neither hepatocellular proliferation nor apoptosis appeared in response to either isoflurane or sevoflurane exposure (Figs. 7a-j).

Discussion

All inhalation anesthetics have been reported to induce hepatic injury after anesthesia in rare cases [1, 2, 7-13], but a clear understanding of the process of anesthetic-induced hepatic injury (necrosis) remains elusive. There have been some reports describing histochemical and immunohistochemical studies of the process of hepatic injury after halothane exposure to rats [14-16].

In the present study, 1.5% isoflurane or 2.0% sevoflurane (approximately 1 MAC) was used for the exposure so that rats could inhale the greatest amount of anesthetics while still breathing naturally. A 2 h exposure

time was adopted because more than 1.5 h is needed to obtain a constant anesthetic concentration according to a report by Hirai [21].

Glycogen and rRNA granules, in the PAS(−) area intracellularly disappear at H 0 and H 6, respectively, and at H 6, AST levels in the blood rose after exposure to each anesthetic. There were 2 patterns of development of the PAS(−) area after anesthetic exposure as follows: the size of the PAS(−) area exposed to isoflurane continued to increase significantly until H 12, while the area exposed to sevoflurane remained almost the same size as that at H 0 until H 12. At H 12, the PAS(−) area had spread the most in cells exposed to the anesthetics in the order of isoflurane > sevoflurane, which is a new finding in the study of isoflurane- and sevoflurane-associated hepatic injury.

In the N-group, the PAS staining temporarily decreased after anesthetic exposure, but the cells in the PAS-decreased area did not become necrotic. In the PB-group, however, PB administration increased the metabolic activity and oxygen demand in the hepatocytes [22]. Only under this accelerated metabolic state, the hepatocytes in the PAS(−) area degenerated to a necrotic state in response to anesthetic exposure in hypoxia. The cells negatively reacted to PAS from the early stage, rRNA disappeared in the PAS(−) area, and infiltrated cells were mostly macrophages. A decrease in systemic as well as hepatic circulation was also previously described to have occurred in animals exposed to anesthetics in hypoxia [14, 21-23]. It is thought that the decreased supply of oxygen to the liver was further induced by oxygen concentrations reaching levels less than 10% in the current study. From these results, it is suggested that severe hypoxia is one of the factors causing necrotic degeneration in hepatocytes.

The appearance of a PAS(−) area and the disappearance of rRNA granules in the PAS(−) area after isoflurane or sevoflurane exposure under 10% oxygen may be attributed to the inhibition of synthesis from DNA to mRNA preventing various syntheses, causing a part of the PAS(−) area to become necrotic. It is not yet clear whether the hepatic injury observed in this model is related to not only hypoxia but also to some active intermediates of anesthetics (which have not yet been specified). This theory is consistent with the reports of Shingu *et al.* [24, 25], who observed an induction of liver injury in rats exposed to halothane or isoflurane in less than 10% oxygen and in less than 10% oxygen

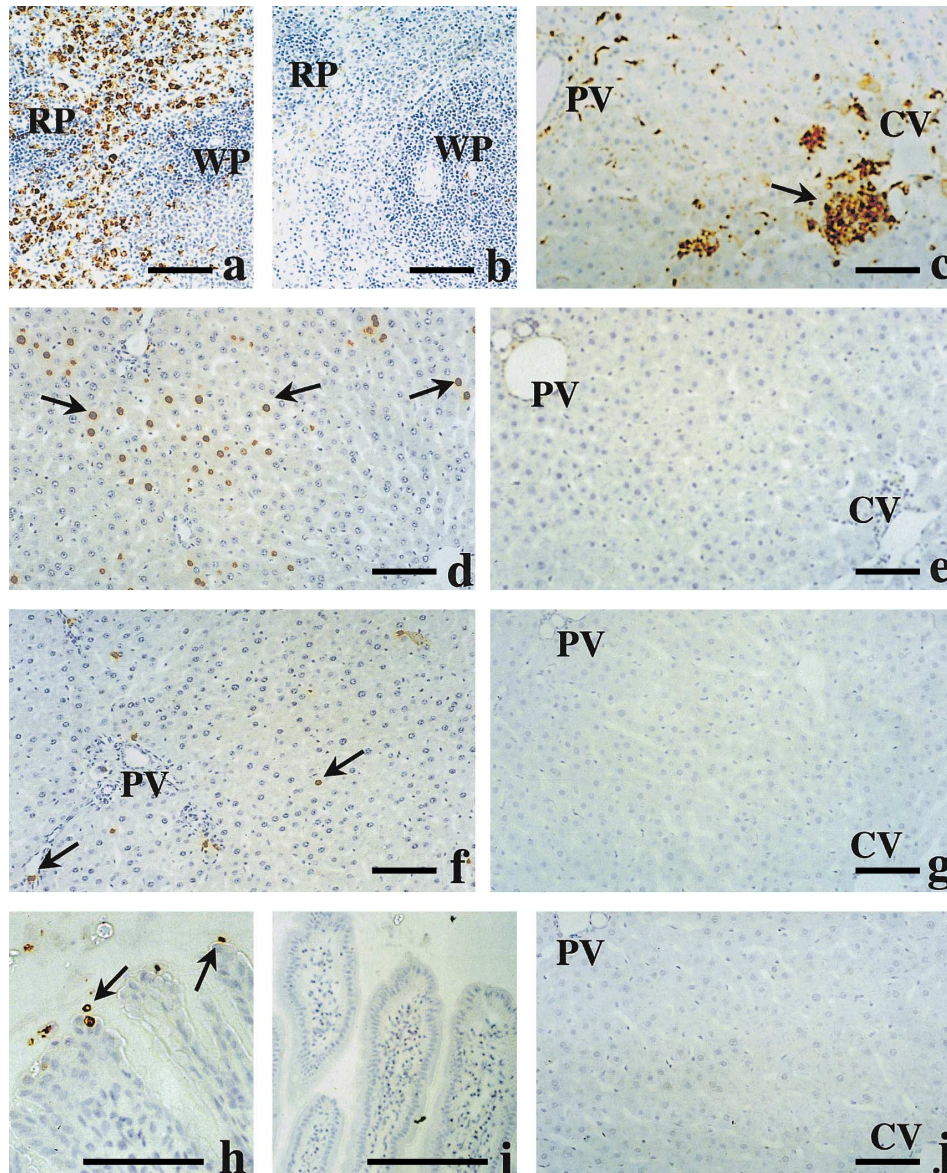


Fig. 7 Images immunohistochemically observed in the PB-group after isoflurane or sevoflurane exposure under 10% oxygen. **a**, The image of the macrophages reacting to anti-macrophage mouse monoclonal antibody (ED I) (positive-control) in the control (normal) rat spleen. Positive reaction to ED I was observed in red pulp (RP) of the spleen. **b**, The image of the negative-control of the photo. **a**. **c**, Image of the infiltrated cells (↑) reacting to ED I around the CV after sevoflurane exposure on Day 1. The image after isoflurane exposure was almost the same as that after sevoflurane exposure. **d**, Image of the positive control of the proliferated cells in control rats (↑) reacting to anti-BrdU mouse monoclonal antibody by liver-excision methods of Higgins *et al.* **e**, Image of the negative-control of the photo. **d**. **f**, Image of the proliferation cells (↑) of the control (normal) rat. BrdU intake was seen sporadically. **g**, The proliferation (BrdU-intake) cell was not observed from H 0 to Day 7 after isoflurane or sevoflurane exposure. The photo. shows an image from Day 1 after sevoflurane exposure. **h**, Image of the positive-control of the apoptotic cells of the intestinal epithelial cells (↑) in the control (normal) rat intaking anti-single stranded DNA (ssDNA) rabbit polyclonal antibody. **i**, Image of the negative-control of the photo. **h**. **j**, Apoptotic cells of the hepatocytes were not observed from H 0 to Day 7 after isoflurane or sevoflurane exposure in this study. The photo. shows the image from Day 1 after sevoflurane exposure. Bars in photos a-j indicate 20 μm. PB-group, see Fig. 1; PAS(−), see Fig. 3; RP, Red pulp of spleen; WP, White pulp of spleen.

alone.

Knights *et al.* [26] have reported the occurrence of mitosis in the liver at H 48 after halothane exposure, but in the study of BrdU-intake until Day 7 showed no hepatocellular regeneration after isoflurane or sevoflurane exposure. The reason why Knights *et al.* noted mitosis may be because they used a milder oxygen condition (14% oxygen concentration) for halothane exposure compared with our severe condition (10% oxygen concentration, with and without anesthetics).

According to Kerr [27], necrosis occurs when hypoxia is severe, whereas apoptosis occurs when hypoxia is mild. In the current study, apoptosis was not observed from H 0 to Day 7 after isoflurane or sevoflurane exposure when we stained liver sections using anti-ssDNA rabbit polyclonal antibody. Our conditions, however, may have been too severe to permit apoptosis.

The current study was carried out by the same method to compare that of the halothane. In rats exposed to halothane in the PB-group, the PAS(−) area covered approximately 56.3% of the area from the CV to the PV in the lobule at H 0, remaining almost the same size until Day 1, then decreasing. After halothane exposure, large vacuoles appeared in the boundary between the PAS(−) area in the CV area where the cells had become necrotic and the PAS(+) area in the PV area. At H12, AST values in the sera exposed to halothane were approximately 230 U.

Based on these results, the area where hepatocytes turned to PAS(−) in response to anesthetic exposure is considered to show the grade of hepatic injury. The grades of both isoflurane- and sevoflurane-induced hepatic injuries were found to be much lower than that of halothane, and that of isoflurane was greater than that of sevoflurane, which is consistent with the data of Yamasaki *et al.* [16]. If the results of the studies of the hepatic circulation in dogs made by Frink *et al.* [14] and Hirai [21] may be applied to the studies on rats, the PAS(−) areas in rat hepatocytes in the current study are likely related to the oxygen supply to the liver. In our results, however, it remains unclear whether appearance of the PAS(−) area and the disappearance of the rRNA are caused by the exposure to these anesthetics or by some other factors. In the N-group, hepatocyte changes are considered to be reversible after each anesthetic exposure in 10% oxygen.

As Rozga *et al.* [28] have described, the recovery from inhalation anesthetic-related necrosis in the CV area

might be due to the necrosis being compressed by and filled with hepatocytes located more peripherally in the lobule than in the necrotic area. As hepatocytes approach the central zone of the liver lobule, neighboring hepatic cell cords move closer to each other, becoming densely packed and compressed, and finally occluded the interposed sinusoidal lumen. The liver reconstruction process observed in the current study appears to be the same as that reported in their study. The authors speculate that in the necrotic area, macrophages appeared from H 6 to Day 2, although neither apoptosis nor proliferation was observed in hepatocytes after isoflurane or sevoflurane exposure, and that the hepatolobular structure was recovered due to the rearrangement of the remaining hepatocytes in the PV area along the hepatic cell cords.

In conclusion, glycogen at H 0 and rRNA at H 6 disappeared from zone 3, and AST levels in the blood rose at H 6 after isoflurane or sevoflurane exposure under 10% oxygen in the PB-group, causing an inhibition of mRNA synthesis and a development of necrosis in the hepatocytes around the CV. There were two patterns of development of the PAS(−) area after isoflurane or sevoflurane exposure: the size of the PAS(−) area exposed to isoflurane kept increasing until Day 1, while that exposed to sevoflurane remained almost the same size until H 12 after the exposure. The grade of hepatic injury induced by isoflurane exposure was higher than that induced by sevoflurane.

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References

1. Brody GL and Sweet RB: Halothane anesthesia as a possible cause of massive hepatic necrosis. *Anesthesiology* (1963) 24: 29–37.
2. Moulton PJA and Sherlock S: Halothane-related hepatitis: A clinical study of twenty-six cases. *Q J Med* (1975) 44: 99–114.
3. Neuberger J, Mieli-Vergani G, Tredger JM, Davis M and Williams R: Oxidative metabolism of halothane in the production of altered hepatocyte membrane antigens in acute halothane-induced hepatic necrosis. *Gut* (1981) 22: 669–672.
4. Kenna JG, Satoh H, Christ DD and Pohl LR: Metabolic basis for a drug hypersensitivity: Antibodies in sera from patients with halothane hepatitis recognize liver neoantigens that contain the trifluoroacetyl group derived from halothane. *J Pharmacol Exp Ther* (1988) 245: 1103–1109.
5. Eger EI II: Isoflurane: A review. *Anesthesiology* (1981) 55: 559–576.
6. Kharasch ED: Biotransformation of sevoflurane. *Anesth Analg* (1995) 81: S27–S38.
7. Stoelting RK, Blitt CD, Cohen PJ and Merin RG: Hepatic dysfunction

- after isoflurane anesthesia. *Anesth Analg* (1987) 66: 147-153.
8. Carrigan TW and Straughen WJ: A report of hepatic necrosis and death following isoflurane anesthesia. *Anesthesiology* (1987) 67: 581-583.
9. Eger EI II, Smuckler EA, Ferrell LD, Goldsmith CH and Johnson BH: Is enflurane hepatotoxic? *Anesth Analg* (1986) 65: 21-30.
10. Paull JD and Fortune DW: Hepatotoxicity and death following two enflurane anaesthetics. *Anaesthesia* (1987) 42: 1191-1196.
11. Shichinohe Y, Masuda Y, Takahashi H, Kotaki M, Omoto T, Shichinohe M and Namiki A: A case of postoperative hepatic injury after sevoflurane anesthesia. *Masui (Jpn J Anesthesiol)* (1992) 41: 1802-1805 (in Japanese with English abstract).
12. Nishiyama T, Yokoyama T and Hanaoka K: Liver and renal function after repeated sevoflurane or isoflurane anaesthesia. *Can J Anaesth* (1998) 45: 789-793.
13. Njoku D, Laster MJ, Gong DH, Eger EI II, Reed GF and Martin JL: Biotransformation of halothane, enflurane, isoflurane, and desflurane to trifluoroacetylated liver proteins: Association between protein acylation and hepatic injury. *Anesth Analg* (1997) 84: 173-178.
14. Frink EJ, Morgan SE, Coetzee A, Conzen PF and Brown BR Jr: The effects of sevoflurane, halothane, enflurane, and isoflurane on hepatic blood flow and oxygenation in chronically instrumented greyhound dogs. *Anesthesiology* (1992) 76: 85-90.
15. Yamada T, Nomura T, Miki Y, Kanda S and Sasaki J: Histochemical and immunohistochemical changes in rat hepatocytes after halothane exposure. *J Anesthesia* (2002) 16: 138-144.
16. Yamasaki A, Takahashi T, Suzuki T, Fujiwara T, Hirakawa M, Ohmori E and Akagi R: Differential effects of isoflurane and halothane on the induction of heat shock proteins. *Biochem Pharmacol* (2001) 62: 375-382.
17. McLain GE, Sipes IG and Brown BR Jr: An animal model of halothane hepatotoxicity: Roles of enzyme induction and hypoxia. *Anesthesiology* (1979) 51: 321-326.
18. Mazze RI, Rice SA and Baden JM: Halothane, isoflurane, and enflurane MAC in pregnant and nonpregnant female and male mice and rats. *Anesthesiology* (1985) 62: 339-341.
19. Tamada M, Inoue T, Watanabe Y, Kawakubo Y, Ogo M, Okumura N, Tamura T and Satoh N: MAC values of sevoflurane. *Progress in Medicine* (1986) 6: 3248-3254 (in Japanese with English abstract).
20. Higgins GM and Anderson RM: Experimental pathology of the liver; Restoration of the liver of the white rat following partial surgical removal. *Arch Pathol* (1931) 12: 186-202.
21. Hirai Y: Effect of isoflurane anesthesia on systemic and hepatic circulation in the dog. *Okayama Igakkai Zasshi (JOMA)* (1987) 99: 1377-1390 (in Japanese with English abstract).
22. Hirayama T, Samuta T, Okada N, Ido Y, Yamada T, Hayashi K, Imaoka S, Funae Y and Kosaka F: Changes in Rat hepatic microsomal cytochrome P-450 caused by the exposure of halothane. *Hiroshima J Anesthesia* (1991) 27: 69-76 (in Japanese with English abstract).
23. MacDonald AC, Marble AE and Perkins JG: Hepatic blood flow and metabolism. *Arch Surg* (1979) 114: 616-622.
24. Shingu K, Eger EI II and Johnson BH: Hypoxia per se can produce hepatic damage without death in rats. *Anesth Analg* (1982) 61: 820-823.
25. Shingu K, Eger EI II, Johnson BH, Van Dyke RA, Lurz FW, Harper MH and Cheng A: Hepatic injury induced by anesthetic agents in rats. *Anesth Analg* (1983) 62: 140-145.
26. Knights KM, Gourlay GK, Hall PD, Adams JF and Cousins MJ: Halothane hepatitis in an animal model: Time course of hepatic damage. *Br J Exp Pathol* (1987) 68: 613-624.
27. Kerr JF: Shrinkage necrosis: A distinct mode of cellular death. *J Pathol* (1971) 105: 13-20.
28. Rozga J, Jeppsson B and Bengmark S: Portal branch ligation in the rat. Reevaluation of a model. *Am J Pathol* (1986) 125: 300-308.